Transgenic Res (2014) 23:477–487
DOI 10.1007/s11248-014-9783-2

ORIGINAL PAPER

Recombinant barley-produced antibody for detection and immunoprecipitation of the major bovine milk allergen, β-lactoglobulin

A. Ritala · S. Leelavathi · K.-M. Oksman-Caldentey · V. S. Reddy · M.-L. Laukkanen

Received: 11 September 2013 / Accepted: 25 January 2014 / Published online: 5 February 2014

Abstract Recombinant allergens and antibodies are needed for diagnostic, therapeutic, food processing and quality verification purposes. The aim of this work was to develop a barley-based production system for β-lactoglobulin (BLG) specific immunoglobulin E antibody (D1 scFv). The expression level in the best barley cell clone was 0.8–1.2 mg/kg fresh weight, and was constant over an expression period of 21 days. In the case of barley grains, the highest stable productivity (followed up to T2 grains) was obtained when the D1 scFv cDNA was expressed under a seed-specific *Glutelin* promoter rather than under the constitutive *Ubiquitin* promoter. Translational fusion of ER retention signal significantly improved the accumulation of recombinant antibody. Furthermore, lines without ER retention signal lost D1 scFv accumulation in T2 grains. Pilot scale purification was performed for a T2 grain pool (51 g) containing 55.0 mg D1 scFv/kg grains. The crude extract was purified by a two-step purification protocol including IMAC and size exclusion chromatography. The purification resulted in a yield of 0.47 mg of D1 scFv (31 kD) with high purity. Enzyme-linked immunosorbent assay revealed that 29 % of the purified protein was fully functional. In immunoprecipitation assay the purified D1 scFv recognized the native 18 kD BLG in the milk sample. No binding was observed with the heat-treated milk sample, as expected. The developed barley-based expression system clearly demonstrated its potential for application in the processing of dairy milk products as well as in detecting allergens from foods possibly contaminated by bovine milk.

Keywords Barley · β-lactoglobulin-specific antibody · Cell culture · D1 scFv · Grain · Recombinant

Electronic supplementary material The online version of this article (doi:10.1007/s11248-014-9783-2) contains supplementary material, which is available to authorized users.

A. Ritala (✉) · K.-M. Oksman-Caldentey · M.-L. Laukkanen
VTT Technical Research Centre of Finland, Tietotie 2, P.O. Box 1000, 02044 VTT Espoo, Finland
e-mail: anneli.ritala@vtt.fi

S. Leelavathi · V. S. Reddy
International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India

Introduction

The most common form of allergy, immunoglobulin E (IgE)-mediated hypersensitivity, affects more than 25 % of the world's population. Hypersensitivity can be defined as an exaggerated and inappropriate immune response to an allergen. Currently, there is a need to produce recombinant forms of several allergens and antibodies for diagnostic, therapeutic, food processing and quality verification purposes. Hitherto

the treatment of food allergy has mainly involved avoidance of the allergen and symptom-specific treatment of allergic reactions and anaphylaxis. Only recently, the research has addressed possibilities to develop therapies that can modify the allergic immune responses in both allergen-specific and non-specific ways (Akdis and Blaser 2000; Majamaa and Isolauri 1997). Allergen-specific immunotherapy includes oral immunotherapy, heat-denatured protein immunotherapy (e.g. application of extensively heated egg and milk diets), sublingual immunotherapy, epicutaneous immunotherapy, and modified recombinant food protein vaccines. These therapies are in various stages of development, the most advanced being in phase 1 and 2 clinical trials (Virkud and Vickery 2012).

Extensive research in plant molecular farming has shown that a wide range of monoclonal antibodies and other pharmaceutically important proteins can be efficiently expressed in plants. The general eukaryotic protein synthesis pathway is very well conserved between plants and animals: Plants can fold and assemble very complex proteins (Vaquero et al. 1999; Schillberg et al. 2003; Negrouk et al. 2005). Proteins of interest accumulate to high levels in plants, and plant-derived molecules are functionally equivalent to those produced in mammalian cell cultures. Plant systems are also advantageous in terms of safety, because plant-produced recombinant proteins are not contaminated with mammalian pathogens or oncogenic sequences, i.e. plants are considered to have generally recognized as safe (GRAS) status (Fischer and Emans 2000; Ma et al. 2003).

The overall aim of this study was to develop a plant-based production system for recombinant β-lactoglobulin (BLG)-specific IgE antibodies. BLG is one of the major allergens in bovine milk. Milk allergy is a very complex disorder. It is most prevalent in early childhood, with reported incidences between 2 and 6 %, and decreases toward adulthood to an occurrence of 0.1–0.5 %. Human milk does not contain BLG, but cow's milk substitution is becoming more and more common in infant diets due to decreased numbers of breastfeeding mothers (Høst 2002). Milk can be considered as a source of primary nutrition and BLG as a protein source for children. Nevertheless, it is generally believed that BLG can be removed from milk without greatly altering the milk properties (Jabed et al. 2012). Recent studies have shown that milk allergic patients can be divided into two groups: heated milk-tolerant and heated milk-reactive ones (Virkud and Vickery 2012). A study of Nowak-Wegrzyn et al. (2008) carried out with 100 milk-allergic patients showed that 75 % of the children with reported milk allergy tolerated heated milk in an oral food challenge. Furthermore, new studies have demonstrated that heated milk-tolerant patients usually develop less severe reactions when exposed to unheated milk, indicating a milder food allergy phenotype (Shreffler et al. 2009; Wanich et al. 2009). In addition, Kim et al. (2011) reported indications that the incorporation of heat-denatured milk proteins into the diet accelerated the development of unheated milk tolerance. These immunotherapeutic effects might therefore be gained via diet modifications. For this purpose it would be useful to be able to process low allergen (e.g. BLG) content milk products which could be safely included in the diet of a patient after heat treatment.

The possibility to use barley cell cultures and barley grains as a production host for BLG-specific IgE antibodies was evaluated. Barley cell cultures served as a preliminary screening tool for obtaining proof-of-concept data to elucidate whether the desired protein could be expressed in the chosen host. Furthermore, with reasonable production levels the cell culture system could offer a contained and traceable production platform for diagnostic, food processing and quality verification applications. Barley grains in turn hold the natural storage properties of grains, which is extremely beneficial in distribution and batch processing of the product (Eskelin et al. 2009). Especially the dormancy properties of grains allow the long-term stability of stored recombinant proteins, and thus it is possible to decouple the processing from the growth and harvest cycles (Boothe et al. 2010). Therefore, barley grains could be utilized as a stock for BLG-specific antibodies for diagnostic, therapeutic, food processing and quality verification applications.

Materials and methods

Contruction of β-lactoglobulin specific IgE antibody expression vector

For the production of a 31 kD recombinant BLG-specific IgE antibody (D1 scFv) (Jylhä et al. 2009, Niemi et al. 2007), a barley codon optimized scFv

Table 1 Gene constructs used in this study for the generation transgenic barley cell cultures and plants

Use	Constructs used in the study
Stable expression in barley cell cultures after nuclear transformation	*UBI-I-ss-D1scFvb-His6-HDEL* with hygromycin selection marker (pD1b02)
Production of transgenic barley plants by nuclear transformation	*UBI-I-ss-D1scFvb-His6-HDEL* with hygromycin selection marker (pD1b02)
	GLUB1-ss-D1scFvb-His6-HDEL with hygromycin selection marker (pD1b03)
	GLUB1-ss-D1scFvb-His6 with hygromycin selection marker (pD1b07)

UBI-I = maize *Ubiquitin* promoter and first intron; *ss* = Arabidopsis basic chitinase signal sequence; *D1scFv* = single chain variable fragment of β-lactoglobulin-specific IgE D1 antibody; *His6* = Histidine tag; *HDEL* = ER retention signal; *GLUB1* = endosperm-specific rice *Glutelin* promoter; *b* refer to codon usage optimization for barley

cDNA was ordered from GenScript. The cDNA sequence of D1 scFv does not contain N-glycosylation sites. The scFv element consisted of cDNAs encoding the *Arabidopsis* basic chitinase signal sequence (Haseloff et al. 1997) to transport the heterologous protein to the ER, D1 scFv with the His-tag and an ER retention sequence, HDEL, surrounded by *Not*I and *Hind*III cloning sites. The synthetic scFv element was cloned to pWBVec8 (kindly provided by CSIRO Plant Industry, Canberra, Australia, Wang et al. 1997, 1998) using *Not*I and *Hind*III sites. The cDNA of D1 scFv was either under control of a constitutive maize *Ubiquitin* promoter and the first intron (*UBI-I*, Christensen and Quail 1996) or under a seed-specific rice *Glutelin* promoter (*GLUB1*, cloning of the *GLUB1* is described in Eskelin et al. 2009). The sequence encoding the *Ubiquitin* promoter was amplified from plasmid pAHC25 (kindly provided by Peter Quail, USDA, Plant Gene Expression Center, Albany, USA) by using the primers *UBII*-5‘ (5′-AGGAACGCGGCCGCTGC AGCGTGACCCGG-3′) and *UBII*-3′ (5′-TCTTTCGC GGCCGCTGCAGAAGTAACACC-3′). The corresponding promoters were then introduced as *Not*I fragments into pWBVec8 complemented with D1 scFv element. In addition, a construct without a sequence encoding the HDEL retention signal was constructed. For this purpose a 5′ oligo SS-scFv barley (5′-AAAGGTGCGGCCGCATGGCTAAGACTAATCT TTTTC-3′) was utilized to amplify the target sequence without the HDEL sequence from the synthetic D1 scFv element. This was cloned to pWBVec8 under *GLUB1* in the same way as described above. The pWBVec8 backbone carried an *HPH* selection marker encoding Hygromycin resistance under 35S promoter, and was utilized as a selection marker for the putative transformants. The constructs listed in Table 1 were introduced to *Agrobacterium tumefaciens*, strain AGL0 (*Rhizobium radiobacter* ATCC BAA-100; Lazo et al. 1991) for gene delivery to barley cells.

Production and maintenance of transgenic barley cell cultures and regeneration of transgenic plants

The *Agrobacterium*-mediated transformation, selection and regeneration of infected barley embryos (*H. vulgare* L. cv. Golden Promise) followed the procedure described in Eskelin et al. (2009). *Agrobacterium* strain AGL0 (Lazo et al. 1991) was used with a co-cultivation period of 3 sdays at +22 °C. The constructs used in transformations are shown in Table 1. The embryos infected in one batch were coded with a combination of an uppercase letter and a number (e.g. A1). After co-cultivation period embryos were cut into 1-2 mm pieces on solid callus induction medium (CIM) supplemented with 2.5 mg/l Dicamba (Duchefa, Haarlem, The Netherlands), 50 mg/l Hygromycin (Sigma-Aldrich, St. Louis, MO, USA) and 150 mg/l Timentin (Duchefa). Three selection rounds were performed to obtain cell lines, and the presence of the transgenes was screened by PCR (see below). The callus clones originating from different embryos were marked with different lowercase letters (e.g. a) and numbered (e.g. a1). The transgenic cell culture clones were maintained on solid CIM (see above) at +22 °C in the dark and subcultured to fresh medium at 4 week intervals. For suspension culture experiments, 0.75 g fresh weight (FW) inoculum was used in 25 ml of liquid CIM medium supplemented with 2.5 mg/l Dicamba and 50 mg/l Hygromycin. The clones were grown in 125 ml Erlenmeyer flasks at +24 °C in the dark on a rotary shaker at 130 rpm (orbital radius 2.5 cm). The suspensions were subcultured in 1 week intervals as described in Ritala et al. (2008) and the growth curve experiments were performed after 2 months when the suspension cultures were well established.

For the regeneration of transgenic barley plants, the viable cell lines (after three rounds of selection) were transferred to regeneration medium (FHG) supplemented with 1 mg/l Benzylaminopurine (Sigma-Aldrich), 50 mg/l Hygromycin and 150 mg/l Timentin. The regenerants were rooted on CIM supplemented with 50 mg/l Hygromycin and 150 mg/l Timentin. The rooted plants were potted in soil mix (Vermiculite:peat:soil, 2:1:1) and grown in a greenhouse (22/13 °C day/night, 19 h light 300 μmol/$m^{2/}$s, 40–50 % relative humidity). The plants were fertilized weekly (Kukkien Y-lannos, Maatalouspalvelu Oy, Finland: Biolan S.M.3, Biolan Oy, Eura, Finland; and Puutarhan täyslannos, Kemira Oy, Espoo, Finland) according to the manufacturer's instructions. The putative transgenic barley plants were screened for the presence of the transgenes by PCR (see below).

PCR analysis of putative transgenic barley cell cultures and plants

Total genomic DNA from putative transformants (calli or leaf material) was isolated using the CTAB method (Murray and Thompson 1980). The primers scFv2BL: 5′-CAGCCTTAGGGAGAGCGGTG-3′ and scFv2BR: 5′-GTGGTGGTGATGGTGGTGCC-3′ were used to amplify a 757-bp DNA fragment of D1 scFv. The PCR reaction contained 100 ng of plant genomic DNA, 100 μM dNTPs, 0.5 mM $MgCl_2$, 1 × Taq DNA polymerase buffer and 1.25 U Taq DNA polymerase (Perkin Elmer Waltham, MA, USA) in a final volume of 50 μl. Thirty-five cycles were performed under the following conditions: 30 s denaturation at 95 °C, 1 min annealing at 51 °C, and 1 min 30 s extension at 72 °C and finally 7 min at 72 °C.

Extraction of total soluble proteins

For the extraction of the total soluble proteins (TSP) the callus, leaf or grain samples were homogenized on ice in 2 ml round-bottomed Eppendorf tubes with a Retsch-Mill using 4 mm diameter stainless steel balls (Retsch, Haan,

Germany) and extraction buffer containing 100 mM sodium ascorbate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 % Tween-20 (v/v), 1 mM EDTA and 1 μg/ml leupeptine in phosphate buffered saline (PBS), pH 7.4. Lysates were clarified twice by centrifugation at 10,000×*g* for 10 min at +4 °C.

Pilot scale extraction of D1 scFv from barley grains was performed in the Retsch-Mill operated with a 50 ml stainless steel cylinder and two 15 mm balls (Retsch). Approximately 50 g of ground grain mass of T2 plants 3249, 3250, 3254 and 3258 was extracted with 125 ml of PBS, pH 7.4 by vortexing on ice for 15 min. The lysate was clarified twice by centrifugation at 6,000×*g* for 15 min at +4 °C. The extraction was repeated overnight with 100 ml of 1× PBS, pH 7.4 supplemented with 0.1 mM PMSF. The total protein contents of the extracts were measured according to the method of Bradford using the Bio-Rad reagent (Bio-Rad, Hercules, USA) with bovine serum albumin (BSA) as standard (Bradford 1976).

ELISA protocol

The binding properties of the barley-produced recombinant anti-BLG D1 scFv were verified by sandwich enzyme-linked immunosorbent assay (ELISA). The sandwich ELISA, where anti-his antibodies were used in the second stage of detection, will also avoid any background levels due to the presence of histidine rich proteins in the crude extracts of barley grains. For this purpose, BLG was immobilized on to StreptaWell plate and used first to capture the recombinant D1 scFv but not the histidine rich proteins. In the second step, anti-his antibodies were used to detect the His-tag containing D1 scFv antibody. In brief, the total proteins from callus, leaf or grain samples were extracted as described above. The wells of StreptaWell plates (Kaivogen, Turku, Finland) were coated with 1 μg biotinylated BLG (nBLG-bio; Sigma-Aldrich) in 100 μl PBS supplemented with 0.5 % (w/v) bovine serum albumin (PBS–BSA) per well for 1 h with shaking at room temperature (RT). After washing with PBS the plant extracts and the standard proteins were added to the wells (100 μl/well) and incubated for 2 h at RT. After the incubation the wells were washed with PBS. The anti-his tag antibody (1:1,000 dilution, Trend Pharmatech, Ave Surrey, B.C. Canada) in PBS–BSA was applied into the wells (100 μl/well), incubated for 1 h at RT and then washed with PBS. After that the secondary antibody, goat anti-mouse IgG (H + L)–AP conjugate (1:1,000 dilution, Bio-Rad) in PBS–BSA was added (100 μl/well), incubated for 1 h at RT followed by the washing step with PBS. The substrate solution containing 2 mg of 4-nitrophenyl phosphate disodium salt (Sigma-Aldrich) in 1 ml of

diethanolamine-$MgCl_2$ buffer (Reagena, Toivala, Finland) was applied to the wells (100 µl/well). The photometric reading was recorded with a Varioskan microtitre plate reader at a wavelength of 405 nm after 1 h and up to 24-h incubation at RT.

Western blot analysis of D1 scFv

For western blot analysis total protein extracts were separated by gel electrophoresis (SDS-PAGE) using 18 % sodium dodecyl sulphate–polyacrylamide gels. Proteins were electrotransferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The detection of D1 scFv was carried out using anti-his tag antibody (1:1,000 dilution). Goat anti-mouse IgG (H + L)–AP conjugate (diluted 1:2,000) was used as secondary antibody, followed by BCIP/NBT colour development substrate (Promega, Madison, WI, USA) reaction.

Purification of D1 scFv

D1 scFv was purified using immobilized metal affinity chromatography (IMAC) first. Hexahistidinyl tag was introduced into the C-terminal end of D1 scFv, thus facilitating the purification by IMAC. Extract (90 ml) from barley grains was diluted 1:3 in binding buffer (10 mM Hepes, 1 M NaCl, 10 % glycerol, 1 mM imidazole, pH 7.4) and incubated for 16 h with 1 ml of Chelating Sepharose Fast Flow (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) charged with Ni^{2+}. The resin was loaded onto a column and washed with 10 bed volumes of binding buffer, 5 bed volumes of wash buffer 1 (10 mM Hepes, 1 M NaCl, 10 % glycerol, 10 mM imidazole, pH 7.4) and 2.5 bed volumes of wash buffer 2 (10 mM Hepes, 1 M NaCl, 10 % glycerol, 20 mM imidazole, pH 7.4). The elution of the bound D1 scFv was carried out using one bed volume of elution buffer (10 mM Hepes, 1 M NaCl, 10 % glycerol, pH 7.4) containing 50, 75, 100, 200 or 500 imidazole. The elution fractions were analyzed on a Coomassie-stained 15 % SDS-PAGE gel and the resulting 100–500 mM imidazole fractions containing the D1 scFv were pooled. The concentrated pool sample was subjected to an additional size exclusion chromatography (SEC) using Superdex 75 HiLoad 16/60 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) connected to an ÄktaPrime system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) via a 5 ml sample loop. The sample was run into the column and washed with PBS at a flow rate of 0.5 ml/min. A total of 160 1.0 ml fractions were collected. The fractions were analyzed on a Coomassie-stained 15 % SDS-PAGE gel. The protein concentration of the pooled fractions was measured by BCA protein assay (Thermo Scientific, Rockford, IL, USA).

Immunoprecipitation of BLG from cow's milk

The immunoprecipitation assay was performed to study whether the purified D1 scFv recognized native BLG from cow's milk. Pasteurized, skimmed cow's milk was centrifuged (20 000 × g, 2 min, +4 °C) and the supernatant was diluted 1:4 with PBS and used for immunoprecipitation. The irreversible and oriented immobilisation of D1 scFv was carried out utilizing His-tag. First the Co-Sepharose matrix was prepared using Chelating Sepharose Fast Flow (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After a washing step with distilled water the Sepharose matrix was rotated with 200 mM $CoCl_2$ for 2 h at RT. Then the BLG-specific D1 scFv was immobilized onto Co-Sepharose (320 µg protein/0.5 ml Sepharose) by incubating in rotation for 18 h at +4 °C. The irreversible immobilisation of the antibody to cobalt was performed by oxidation with 30 % H_2O_2 for 4 h at RT. Finally the Co-Sepharose was washed as follows: with distilled water, 0.5 M imidazole-PBS, pH 7.4, distilled water, 50 mM EDTA, pH 8.0, distilled water and finally with PBS, pH 7.4.

In the immunoprecipitation assay both heat-treated (incubation at +95 °C) and native cow's milk samples were used. These milk samples were then incubated with D1 scFv-Sepharose matrix for 1 h at RT. After the washing step with PBS-0.05 % Tween 20, elution was performed by adding 50 µl 0.1 M glycine–HCl (pH 2.5) and elution fractions were collected. Neutralization of the fractions was carried out with 1 M Tris–HCl, pH 8.5. The fractions were analyzed on a silver-stained 15 % SDS-PAGE gel.

Results

Transgenic barley cell cultures expressing BLG-specific IgE antibody (D1 scFv)

Altogether 21 transgenic barley cell culture clones carrying the construct pD1b02 were obtained as

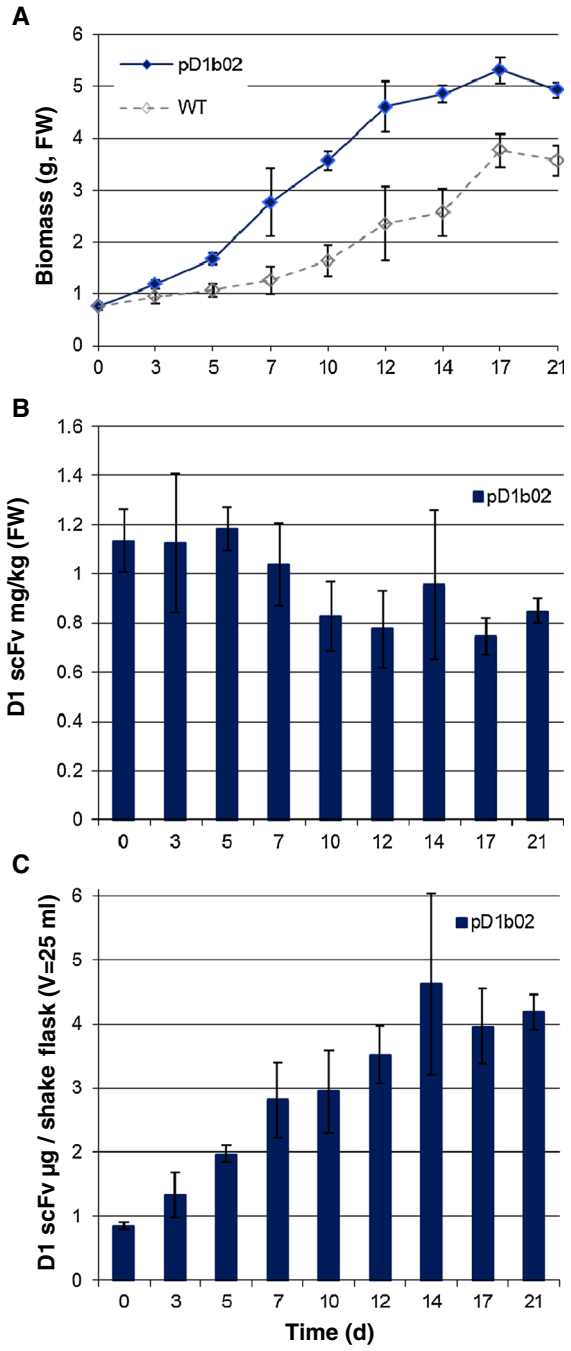

Fig. 1 Growth behaviour and D1 scFv accumulation of the best producing barley cell culture clone (A1/p2). **a** Growth curve, **b** accumulation of D1 scFv in mg/kg (FW) and **c** total D1 scFv yield/shake flask (µg; V = 25 ml) over the culturing period of 21 days. The background level of non-transgenic (WT) level in D1 scFv ELISA assay was close to zero and thus omitted from **b** and **c**

verified by PCR (data not shown). The accumulation of D1 scFv in the established barley cell clones was analyzed by ELISA and western blotting. Eleven of the analyzed barley cell clone extracts exhibited binding to biotinylated BLG in ELISA assay. The first screening of seven day plate-grown material revealed that the overall accumulation level was low, at best reaching 0.23 mg of recombinant D1 scFv in one kilogram of transgenic barley cells (FW) (data shown in Online Resource 1). The best producing barley cell clone (A1/p2) was grown in suspension culture over 21 days and the accumulation of D1 scFv was followed (Fig. 1). The accumulation level was in the range of 0.8 – 1.2 mg/kg (FW), being on average 0.96 ± 0.22 mg D1 scFv per kg cells (FW). The accumulation level was constant over the whole growth period of 21 days. The optimal phase for harvesting with maximum yield was after 14 days of culture when the culture reached the stationary phase.

Transgenic barley plants expressing D1 scFv

The constructs used in the genetic engineering experiments aiming at production of transgenic barley plants are shown in Table 1. A summary of the transformation outcome is shown in table in Online Resource 2.The plants carrying pD1b02 and constitutively expressing the D1scFv originated from five different embryos and thus at least five independent integration events had occurred. In the pD1b03- and pD1b07-carrying plants in which the D1 scFv was expected to be expressed during maturation of the grains, the transgenic plants originated from eight and 15 embryos, respectively, thus indicating at least the same number of integration events. Plants originating from the same transformation event were therefore handled as separate transgenic lines.

The mature grains of T0 plants were harvested individually. All the plants were fertile. The weight of 100 grains and the total grain-set (yield) were measured (data shown in table in Online Resource 3). After harvest the grains were stored at 14 °C. The total grain yield of the plants carrying pD1b07 was significantly lower when compared to the non-transgenic plants regenerated with the same procedure as transgenic plants (Anova single factor, $P = 0.00003$). In addition, the weights of 100 grains of the pD1b03- and

pD1b07-carrying plants were significantly lower when compared with the control plants (Anova single factor, $P = 0.0003$ and $P = 0.0000003$, respectively).

In order to analyze the accumulation of D1 scFv in transgenic T1 grains a half-grain analytical procedure of the non-embryo part was developed. It was found that half grains (embryo parts) can be stored at +14 °C for 1 month without loss of germination capacity (data shown in table in Online Resource 4). This substantially eased the pressure to perform the assays, since all the lines could be analyzed before making the decision of which embryo halves should be propagated for the next generation without sacrificing the ability of the embryos to germinate. In addition, the grinding and extraction of the total proteins from barley grain halves was evaluated for a sufficient analytical procedure. Different soaking (A-C) and milling (1-3) strategies were applied: A = 18 h at + 4 °C in extraction buffer; B = 18 h at −20 °C in extraction buffer; C = dry grains kept 18 h at + 4 °C; 1 = milling with Retsch 2 × 3 min; 2 = milling with Retsch 2x3 min with sand; 3 = milling with Retsch 3x3 min with sand. Furthermore, a second extraction was performed by soaking the once extracted grain residues for 1 h at +4 °C in extraction buffer and by milling with Retsch 1x3 min. The efficacy of the extraction was estimated on the basis of the extracted share of the theoretical protein content of barley grains (10 %). The results are shown in figure in Online Resources 5. The soaking for 18 h in the extraction buffer was beneficial for protein extraction from the grains. The addition of sand and longer milling times (2, 3) did not increase the extraction efficacy and thus they were omitted. Therefore, soaking at +4 °C in extraction buffer and milling with Retsch for 2 × 3 min were chosen for routine procedure (1A). However, the extraction was repeated since an additional yield of approximately 5 % of the theoretical protein content was obtained. In total approximately 25 % of the theoretical grain protein content was extracted with this procedure. On this basis ten half-grains (T1 grains) per T0 plant were screened and from the best expressing grains the T1 generation plants were grown and T2 grains harvested.

Altogether 97 transgenic T0 plants carrying the pD1b02, pD1b03 and pD1b07 constructs were screened for D1 scFv accumulation by a half-grain analytical procedure as described above. No background accumulation was recorded from the non-transgenic control plants (data not shown). Thus the non-expressing individual half grains were regarded as non-transgenic progeny resulting from Mendelian segregation. The best accumulation levels were observed in halved grains carrying the construct pD1b03 having the seed-specific *Glutelin* promoter, *Arabidopsis* basic chitinase signal sequence and HDEL to transport and retain the D1 scFv in the ER (Fig. 2). The best expressing grain halves originated from two different T0 plants (3013, 3042) having same integration event background (B6/i) and more than 10 half grains were analyzed from these plants. The average accumulation level in T1 half grains of T0 plant 3013 was 1.4 ng D1 scFv/mg (n = 29) and in T0 plant 3042 0.9 ng/mg (n = 27). However, the variation between grains was substantial and the best half-grains accumulated D1 scFv in the range of 1.7 – 5.5 ng/mg (Fig. 2).

The embryo parts of the best expressing T1 grains were germinated to obtain the T1 generation plants carrying the T2 grains. A germination frequency of 85.7 % (54/63) was recorded in transgenic lines, whereas the corresponding figure in non-transgenic lines was 90.0 % (9/10). The accumulation level of D1 scFv was followed to T1 generation and analyzed by ELISA from individual T2 grain halves. From some of the best lines whole grains were also analyzed in order to estimate the accuracy of the analysis based on half grain assays. The accumulation levels are shown in Fig. 3. The half grain data gave up to twofold underestimates when compared to whole grain data. At best, the D1 scFv accumulation was on average in the range of 1–2.7 ng/mg of whole grains, the highest level recorded being 3.8 ng/mg grain. The accumulation in grains carrying the pD1b02 and pD1b03 constructs was stable to T2 grains, whereas the pD1b07 lines lost their ability to accumulate D1 scFv.

Purification and functionality of the barley grain-produced D1 scFv

A pilot scale extraction was performed for a batch of pooled (51.0 g) T2 grains. The resulting crude extract indicated in ELISA an accumulation level of 55.0 mg D1 scFv/kg in T2 grains. The D1 scFv was purified from the crude extract by applying a two-step chromatographic purification protocol including IMAC and SEC. The purification resulted in a yield of 0.47 mg (i.e. 9.5 mg/kg grains) of D1 scFv (31 kD) with high purity on the basis of Coomassie stained SDS-PAGE gel (Fig. 4). Furthermore, the ELISA

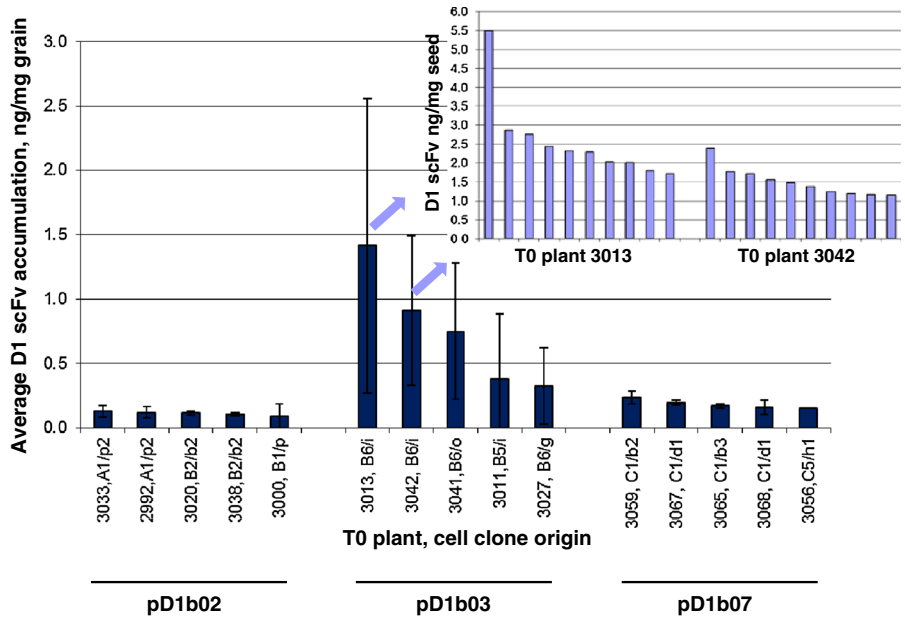

Fig. 2 Average D1 scFv accumulation in the T1 half grains of the top five T0 plants carrying the pD1b02 (*UBI-I-ss-D1scFvb-His6-HDEL*), pD1b03 (*GLUB1-ss-D1scFvb-His6-HDEL*) and pD1b07 (*GLUB1-ss-D1scFvb-His6*) constructs. The small window shows the distribution of accumulation levels in the ten best half-grains of T0 plants 3013 and 3042 in descending order

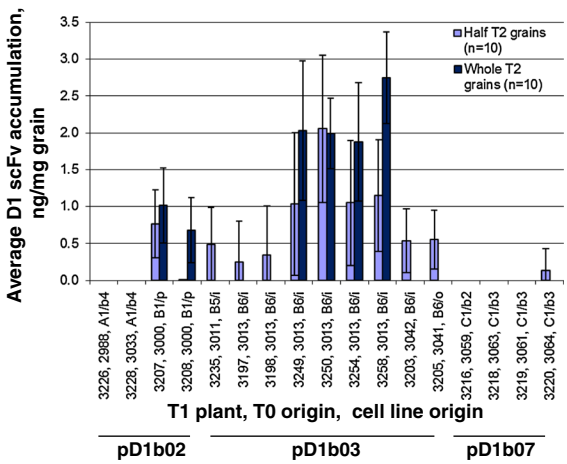

Fig. 3 The average D1 scFv accumulation level in the T2 grains carrying pD1b02 (*UBI-I-ss-D1scFvb-His6-HDEL*), pD1b03 (*GLUB1-ss-D1scFvb-His6-HDEL*) and pD1b07 (*GLUB1-ss-D1scFvb-His6*) constructs analyzed from grain halves (all) and whole grains of T1 plants (3207, 3208, 3219, 3249, 3250, 3254, 3258)

revealed that 29 % of the purified protein (i.e. 2.7 mg/kg grains) was fully functional.

The binding properties of the purified D1 scFv were further characterized by immunoprecipitation assay. The D1 scFv fragment recognized the native BLG with a molecular weight of 18 kD in the milk sample without heat treatment, whereas no binding was observed with the heat-treated milk sample (Fig. 5).

Discussion

The transformation frequency of barley embryos varied between 5 and 10 % when measured as the percentage of embryos giving rise to transgenic barley plants. For research purposes this frequency is acceptable, but for industrial applications the approach should be further optimized to reach a success rate of 25 % as in the case of commercial scale operations at OrfGenetics (www.orfgenetics.com, Einar Mäntylä, personal communication. Transformation efficiency varies strongly due to factors related to environment, personnel, quality of the donor plants and the DNA sequence to be transferred. The average records are ranging from 20 to 25 % but even up to 86 % transformation efficiencies have been reported (Hensel et al. 2007; Bartlett et al. 2008). The accumulation level of BLG-specific antibody (D1 scFv) was modest in transgenic barley cell cultures. However, the

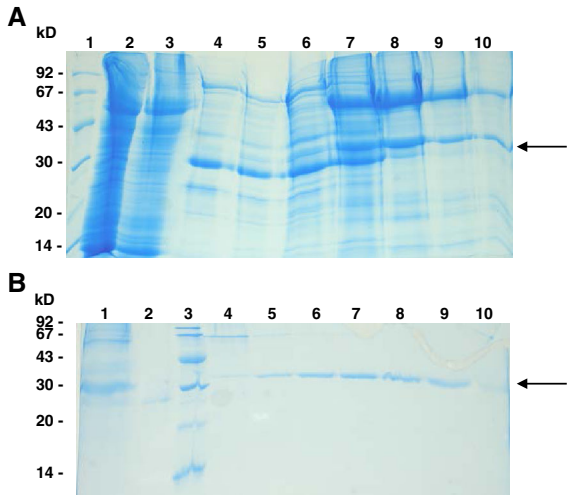

Fig. 4 Purification of D1 scFv from barley grains. **a** After IMAC purification the elution fractions were analyzed on Coomassie stained 15 % SDS-PAGE gel. *Lanes 1* LMW; *2* grain extraction; *3* flow through; *4* wash buffer 1; *5* wash buffer 2; *6* 50 mM imidazole elution; *7* 75 mM imidazole elution; *8* 100 mM imidazole elution; *9* 200 mM imidazole elution; *10* 500 mM imidazole elution. **b** After SEC the fractions were analysed on Coomassie stained 15 % SDS-PAGE gel. *Lanes 1* Sample subjected to SEC; *2* flow through; *3* LMW; *4–10* different fractions from SEC. The *arrow* indicates the D1 scFv protein band

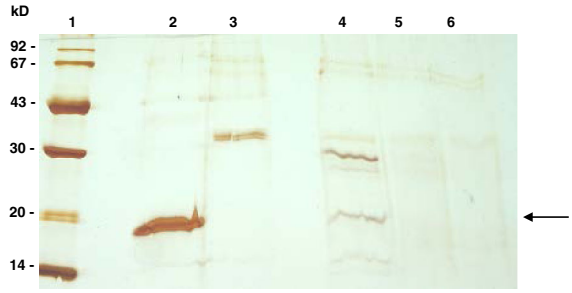

Fig. 5 Immunoprecipitation of BLG from cow's milk using D1 scFv. The purified D1 scFv was immobilized onto the Co-Sepharose matrix and incubated with the milk samples with or without heat treatment at +95 °C for 60 min. The samples were analyzed on a silver-stained 18 % SDS-PAGE gel. *Lanes 1* LMW; *2* commercial BLG; *3* purified D1 scFv; *4* native milk sample with D1 scFv-Co-Sepharose matrix; *5* milk sample after the heat treatment with D1 scFv-Co-Sepharose matrix; *6* No milk sample with D1 scFv-Co-Sepharose matrix. The *arrow* indicates the BLG molecule

accumulation level was six fold higher than was recorded with the same platform for recombinant full-length collagen (Ritala et al. 2008), when a level of 0.14 mg/kg cells (FW) was obtained. The whole plant studies in turn revealed that D1 scFv accumulation interfered with grain filling. This was indicated by grain yield and grain weight rates of T0 plants, especially when the ER retention signal was omitted. This observation suggests that the ER targeted accumulation of antibody somehow overcame some of the unintended pleiotropic effects seen with the non-targeted accumulation. There could be several reasons for such pleotropic effects. The non-targeted accumulation could be interfering in the accumulation of seed storage proteins or the antibody might be interacting with the process involved in the grain developmental processes itself. However, more work is required in future to understand this phenomenon. However, the highest D1 scFv accumulation levels were recorded with the seed-specific *Glutelin* promoter, *Arabidopsis* basic chitinase signal sequence and HDEL retention signal when compared to constitutive expression lines. Interestingly, the lines expressing the construct pD1b07 missing the HDEL retention signal clearly had a lower accumulation level in the half grains when compared to results obtained with the construct carrying the HDEL signal (pD1b03). Furthermore, the pD1b07 lines lost the capacity for D1 scFv accumulation in T2 grains. This data would thus indicate that ER retention due to the HDEL sequence would be beneficial for the stability of the heterologous protein, in accordance with some other published reports (Stöger et al. 2002; Ma et al. 2003; Vitale and Hinz 2005).

Finding suitable grinding and extraction conditions for barley half grains was a challenging task. Extraction is the key step in the recovery of proteins, as shown by Azzoni et al. (2002). They studied extensively the choice of extraction media: which buffers, salts, solvents, and protease inhibitors to include and in what chemical and physical conditions: pH, ionic strength, temperature, time and agitation, in order to achieve good extraction yields with minimum effort and costs. In this study, the aim was to obtain comparable and reliable results with barley half grains in order to be able to develop the best lines for propagation and further studies. The chosen procedure of two extraction rounds resulted in a recovery rate of approximately 25 % of the theoretical grain protein content. This was found to be acceptable for the purpose, although the whole grain extractions revealed that two fold higher yields could be expected. The observed difference might be partly explained by the

unequal halving of the grain. It was taken care that the embryo part was not harmed and it might be that the percentage value share of endosperm in distal grain half was less when compared to a whole grain. Even though the half-grain assay turned out to be inaccurate it can be utilized as a prescreening protocol before the germination of the next generation of plants. Furthermore, the pilot batch grinding and extraction system proved to be very efficient, giving rise to recovery levels of 55.0 mg D1 scFv/kg grains. The corresponding figure with corn was 0.64 mg recombinant aprotinin from 125 g of grains, i.e. 5.1 mg/kg grains (Azzoni et al. 2002) when using material having an accumulation level of 0.17 %.

In this study, functional D1 scFv was successfully expressed in and purified from barley grains. The purified barley-produced D1 scFv was of expected size (31 kD) and recognized the native BLG, whereas no binding with heat-treated milk samples was observed. Previously, we have shown that the BLG binding of bacterially produced D1 Fab fragment was abolished by a prolonged heat treatment, whereas binding to protein bands with molecular weight of about 32 kD appeared (Jylhä et al. 2009). The immunoprecipitated protein bands were identified by MS and it was confirmed that the 18-kD protein precipitated by the Fab fragments was bovine BLG and the 32-kD protein band was α-S1-casein.

The complete removal of allergens from foods is very difficult, or even impossible. However, the production and/or manufacturing of hypoallergenic forms, i.e. products with low or modified allergen contents, is very important for nutritional and safety reasons. In the case of BLG, Jabed et al. (2012) made a very important step forward by introducing the targeted tandem microRNA to dairy cattle, resulting in a 98 % decrease of BLG in milk. Importantly, this was achieved with animals carrying a hemizygous transgenic locus and was not dependent on homozygocity. The decrease in BLG content caused an increase in other milk proteins, revealing a complex balance of synthesis of these proteins. In this particular case, the absence of BLG induced high casein contents and thus a favorable outcome, since increased calcium and high cheese yields were expected. However, casein is also one of the major allergens in cow′s milk and the change induced might not be favorable. It is important to develop other means for removing and/or modifying allergens from foods.

In conclusion, safe and validated recombinant allergens and antibodies are needed for diagnostic, therapeutic food processing and quality verification purposes. We have developed barley-based production systems for BLG-specific IgE antibody (D1 scFv). Barley cell cultures served as a proof of concept, and reasonable D1 scFv levels were attained in barley grains with a glutelin-specific promoter combined with ER targeting and retention signals. The grain-produced D1 scFv was successfully purified using affinity-based chromatographic protocols and the functionality was confirmed. These results will pave the way, not only for the production of BLG-specific antibodies for large scale application but for the production of other recombinant proteins with applications in human and animal health in the future.

Acknowledgments We thank Kristiina Takkinen for fruitful discussions, Armi Boman, Sirkka-Liisa Kanervo, Annika Majanen, Anja Pallas, Jaana Rikkinen and Tuuli Teikari for excellent technical assistance and Michael Bailey for language revision. This work was supported by the Academy of Finland (decision 121595) and Indian Department of Biotechnology (No. BT/IN/Finnish/15/VSR/2007) and EU-COST FA0804 Action 'Molecular farming: plants as a production platform for high value proteins'. The authors declare no conflicts of interests.

References

Akdis CA, Blaser K (2000) Mechanisms of allergen-specific immunotherapy. Allergy 55:522–530

Azzoni AR, Kusnadi AR, Miranda EA, Nikolov ZL (2002) Recombinant aprotinin produced in transgenic corn seed: extraction and purification studies. Biotech Bioeng 80:268–276

Bartlett JG, Alves SC, Smedley M, Snape JW, Harwood WA (2008) High-throughput *Agrobacterium*-mediated barley transformation. Plant Methods 4:22. doi:10.1186/1746-4811-4-22

Boothe J, Nykiforuk C, Shen Y, Zaplachinski S, Szarka S, Kuhlman P, Murray E, Morck D, Moloney MM (2010) Seed-based expression systems for plant molecular farming. Plant Biotech J 8:588–606

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Christensen AH, Quail PH (1996) *Ubiquitin* promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218

Eskelin K, Ritala A, Suntio T, Blumer S, Holkeri H, Wahlström EH, Baez J, Mäkinen K, Nuutila AM (2009) Production of a recombinant full-length collagen type I α-1 and of a

45-kDa collagen type I α-1 fragment in barley seeds. Plant Biotech J 7:657–672

Fischer R, Emans N (2000) Molecular farming of pharmaceutical proteins. Transgenic Res 9:279–299

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. Proc Natl Acad Sci USA 94:2122–2127

Hensel G, Valkov V, Middlefell-Williams J, Kumlehn J (2007) Efficient generation of transgenic barley: the way forward to modulate plant–microbe interactions. J Plant Physiol 165:71–82

Høst A (2002) Frequency of cow's milk allergy in childhood. Ann Allergy Asthma Immunol 89:33–37

Jabed A, Wagner S, McCracken J, Wells DN, Laible G (2012) Targeted microRNA expression in dairy cattle directs production of β-lactoglobulin-free, high-casein milk. Proc Natl Acad Sci USA 109:16811–16816

Jylhä S, Mäkinen-Kiljunen S, Haahtela T, Söderlund H, Takkinen K, Laukkanen M-L (2009) Selection of recombinant IgE antibodies binding the β-lactoglobulin allergen in a conformation-dependent manner. J Immunol Meth 350:63–70

Kim JS, Nowak-Wegrzyn A, Sicherer SH, Noone S, Moshier EL, Sampson HA (2011) Dietary baked milk accelerates the resolution of cow's milk allergy in children. J Allergy Clin Immunol 128:125–131

Lazo GR, Stein PA, Ludwig RA (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. Bio/Tech 9:963–967

Ma JK, Drake PM, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794–805

Majamaa H, Isolauri E (1997) Probiotics: a novel approach in the management of food allergy. J Allergy Clin Immunol 99:179–185

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucl Acids Res 8:4321–4325

Negrouk V, Eisner G, Lee H, Han K, Taylor D, Wong HC (2005) Highly efficient transient expression of functional recombinant antibodies in lettuce. Plant Sci 169:433–438

Niemi M, Jylhä S, Laukkanen M-L, Söderlund H, Mäkinen-Kiljunen S, Kallio JM, Hakulinen N, Haahtela T, Takkinen K, Rouvinen J (2007) Molecular interactions between a recombinant IgE antibody and β-lactoglobulin allergen. Structure 15:1413–1421

Nowak-Wegrzyn A, Bloom KA, Sicherer SH, Shreffler WG, Noone S, Wanich N, Sampson HA (2008) Tolerance to extensively heated milk in children with cow's milk allergy. J Allergy Clin Immunol 122:342–347

Ritala A, Wahlström EH, Holkeri H, Hafren A, Mäkeläinen K, Baez J, Mäkinen K, Nuutila AM (2008) Production of recombinant industrial protein using barley cell cultures. Protein Expr Purif 59:274–281

Schillberg S, Fischer R, Emans N (2003) Molecular farming of recombinant antibodies in plants. Cell Mol Life Sci 60:433–445

Shreffler WG, Wanich N, Moloney M, Nowak-Wegrzyn A, Sampson HA (2009) Association of allergen-specific regulatory T cells with the onset of clinical tolerance to milk protein. J Allergy Clin Immunol 123:43–52

Stöger E, Sack M, Fischer R, Christou P (2002) Plantibodies: applications, advantages and bottlenecks. Curr Opin Biotech 13:161–166

Vaquero C, Sack M, Chandler J, Drossard J, Scuuster F, Monecke M, Schillberg S, Fischer R (1999) Transient expression of a tumor-specific single chain fragment and a chimeric antibody in tobacco leaves. Proc Natl Acad Sci USA 96:11128–11133

Virkud YV, Vickery BP (2012) Advances in immunotherapy for food allergy. Discov Med 14:159–165

Vitale A, Hinz G (2005) Sorting of proteins to storage vacuoles: How many mechanisms? Trends Plant Sci 10:316–323

Wang MB, Upadhyaya MN, Brettell RIS, Waterhouse PM (1997) Intron mediated improvement of a selectable marker gene for plant transformation using *Agrobacterium tumefaciens*. J Genet Breed 51:325–334

Wang MB, Matthews PR, Upadhyaya MN, Waterhouse PM (1998) Improved vectors fro *Agrobacterium tumefaciens*-mediated plant transformation. Acta Hort 461:401–407

Wanich N, Nowak-Wegrzyn A, Sampson HA, Shreffler WG (2009) Allergen-specific basophil suppression associated with clinical tolerance in patients with milk allergy. J Allergy Clin Immunol 123:789–794